Amendments to the Specification

Please add the following new paragraph after paragraph [0026]:

-- Figures 9A through 9C depict embodiments of the tangential flow filtration device for the separation of leukocytes and also monocytes from a blood product sample. Figure 9A provides an embodiment of the device for the enrichment of leukocytes wherein the cross-flow chamber is above the filtration chamber. Figure 9B depicts a front view of the device wherein the input of sample is below the filter and the filtrate passing upward through the filter for the enrichment of monocytes. Figure 9C is an overhead view of the device depicted in Figure 9B. --

Please replace paragraph [0038] with the following amended paragraph:

-- In another method for enriching a cell population for monocytic dendritic cell precursors from a sample of blood constituents provides for tangential flow filtration of the leukocytes from cellular debris, red blood cells and other cells and particles in a blood sample. A description of the device and its use is described in WO2004/000444, incorporated herein by reference in its entirety. The method comprises (1) introducing the blood sample into a tangential flow filtration (TFF) unit, the TFF unit comprising a cross-flow chamber, a filtrate chamber, and a filter in fluid communication with the cross-flow chamber and the filtrate chamber, the filter having a pore size of about 1 to about 10 microns, typically about 5.5 microns; (2) recirculation of the sample through the TFF unit at a predetermined input rate, typically about 1400 ml/min, and a predetermined filtration rate, typically about 15 to about 21 ml/min, more typically about 17 ml/min, the predetermined input rate at least five times the predetermined filtration rate; wherein the predetermined filtration rate is less than the unopposed filtration rate for the filter; and (3) isolating a cell population enriched for leukocytes. Typically the filtration time is about 60 to about 90 minutes. The method can result in an enriched cell population that is substantially free of non-leukocyte blood constituents including plasma, platelets and erythrocytes. The enriched cell population produced by this method can comprise

at least about 50% monocytic dendritic cell precursors and preferentially at least about 70% monocytic dendritic cell precursors that have not been activated. The method can further comprise the collecting of blood from a subject and preparing the sample from the blood by leukapheresis, density centrifugation, differential lysis, filtration, or preparation of a buffy coat prior to tangential flow filtration. Performing the TFF purification of the monocytic DC precursors at room temperature, or below (*i.e.*, below 37 °C) further aids in reducing the activation of the cells. The TFF procedure is described further herein (*see* "Tangential Flow Filtration," *infra*). --

Please add the following new paragraphs after paragraph [0044]:

-- Tangential Flow Filtration

Tangential flow filtration devices and methods can be used for processing a heterogeneous mixture of blood constituents to provide an enriched population of leukocytes. In one aspect in accordance with the present invention, tangential filtration flow devices and methods are provided for the enrichment of monocytes by the selective removal of non-monocyte blood constituents, including, for example, the removal of lymphocytes, erythrocytes, platelets and the like from the mixture.

The terms "tangential flow filtration" and "cross-flow filtration" are used interchangeably and refer to the separation of suspended particles (*e.g.*, cells) from a fluid mixture, including the separation of particles of a defined characteristic (*e.g.*, a desired size range) from a heterogeneous mixture of particles in the fluid mixture. The particles are separated by passing or circulating the fluid mixture (*e.g.*, a sample fluid) in a sample chamber substantially parallel or tangential to a filter (*e.g.*, the surface of the filter facing the sample fluid), typically under some positive pressure, with the fluid mixture comprising the concentrated particles, or leukocytes, continuing to flow tangential to the membrane surface.

Generally, determination of which particles are removed in the "filtrate," *i.e.*, that portion of fluid passing through the filter, and those particles retained in the "retentate" is

dependent on a variety of factors. Such factors include, *e.g.*, filter pore size, input rate, filtration rate, concentration of particles in the fluid mixture, temperature, and viscosity of the fluid mixture. As used herein, "pore size" refers to the average size of the pores in the filter. "Input rate" refers to the rate at which a sample (*e.g.*, fluid mixture) is introduced into the chamber housing the filter. Where the sample is recirculated multiple times across a filter (*e.g.*, in a device according to the present invention), the input rate is also referred to as the "recirculation rate." "Cross-flow" refers to the substantially parallel (*i.e.*, parallel to the surface of the filter in any direction) flow of the fluid mixture across the filter. "Cross-flow rate" refers to the rate of flow of sample, or fluid mixture, over and substantially parallel to the filter; the cross-flow rate of the fluid mixture is generally dependent on a variety of parameters, including, for example, the input rate and the size and shape of the chamber housing the filter. "Filtration rate" refers to the rate of flow of the fluid mixture through the filter. The filtration rate for a device and the methods according to the present invention is typically less than the unopposed (*i.e.*, open tube) filtration rate. "Output rate" refers to the rate of removal of the fluid mixture from the cross-flow chamber, other than the fluid mixture passing through the filter (*i.e.*, the filtrate). The output rate is generally equal to the input rate minus the filtration rate.

As used herein, the term "filter" refers to any article made of any material or combination of materials having a plurality of pores that allow one or more components (*e.g.*, blood constituents) of a sample or fluid mixture subjected to cross-flow across the article to pass through it, thereby separating those components (*e.g.*, non-leukocytes) from other components (*e.g.*, leukocytes). The surface of a filter can have any suitable area, such as, for example, about 42 to about 145 mm in diameter, although filters of greater and lesser area can be used. In certain embodiments, only one filter is used in a TFF device. In other embodiments, additional filters can be used in a TFF device.

The filter typically employed in the TFF device of the present invention can be chosen from a wide range of organic polymeric filters. Such filters include, but are not limited to, microporous membranes of nylon, polyvinylidene fluoride (PVDF), cellulose acetate/nitrate, polysulfone, polycarbonate, polyethylene, polyester, polypropylene, and polyamide. Other

filters, such as ceramic filters and metallic filters, can also be used. Both hydrophilic and hydrophobic, charged and uncharged filters can be used. In certain applications, hydrophilic filters can be preferred.

A filter of the tangential filtration flow device typically comprises a number of pores distributed across the area of the filter. In certain embodiments, the filter has a pore size with a small variation in pore size. For example, the variability in the pore size can be about ± 20%, or within the range of about + 0 to about 20%. In a typical embodiment, "nuclepore" or "track etched" filters are used (*e.g.*, Poretics® polyethylene or polycarbonate track-etched filter membranes (Osmonics, Minnetonka, MN)). These filters typically have a smooth surface with tightly controlled pore sizes in the material. Such filters are typically prepared by exposing a flat sheet of non-porous plastic to a source of radioactive particles, which are energetic enough to pierce to plastic sheet. The "tracks" are then enlarged in diameter by exposure to chemical solvents or etching agents. The size of the pores can be controlled by the track etching conditions.

Tangential flow filtration takes advantage of differences between various cell types in blood to enrich for leukocytes (*e.g.*, monocytes, dendritic cell precursors, and the like). Such differences can include, *e.g.*, differences in size, shape and/or deformability. The size and deformability of cells in human blood typically varies by cell type. Erythrocytes (red blood cells) are typically biconcave disk shaped, enucleate, measure about 7 microns in the major diameter and are relatively deformable. Polymorphonuclear leukocytes cells are typically spheroidal, also about 7 microns, but less deformable than erythrocytes. Of the mononuclear cells, lymphocytes are typically 7 to 10 microns, and monocytes usually are in the range of 10 to 15 microns.

In various embodiments, the filter pore size is selected to enrich for leukocytes, and/or to fractionate blood constituents, thereby enriching for leukocytes. For example, in certain embodiments, monocytes having a nominal diameter of 10 to 15 microns, and erythrocytes having a nominal diameter of 7 microns, can be separated by TFF using a filter having a pore size of about 5 to about 5.5 microns. In a particular embodiment a filter of 4.5

microns was used to successfully separate monocytes from the other cellular constituents of a leukopheresis sample.

In other embodiments, the filter pore size can be within the range of about 1 to about 10 microns, or about 3 to about 8 microns, or about 3 to about 5 microns. A filter pore size in the range of about 3 microns can retain most leukocytes, and effect less efficient removal of erythrocytes from the leukocytes. In contrast, a filter pore size in the range of about 8 microns can effect more efficient removal of erythrocytes, but increases the loss of leukocytes in the filtrate. A filter size of about 3 to about 5.5 microns can be used to enrich for $CD34^+$ hematopoietic stem cells.

The enrichment of leukocytes from other cellular blood constituents can also be affected by the input rate, the filtration rate, and/or the concentration of cells in the sample or fluid mixture. For example, erythrocytes are more deformable than leukocytes and can, therefore, be more readily passed through a filter pore size smaller than the major diameter of the erythrocytes (*e.g.*, less than about 7 microns). In a specific example, erythrocytes can be separated from leukocytes using filters having pore size of about 5 microns. In other embodiments, the filter pore size is decreased to about 3 microns, and the concentration of cells increased (*supra*) to efficiently separate erythrocytes from leukocytes.

The enrichment of leukocytes from other cellular blood constituents can also be effected by maintaining a filtration rate that is less than the unopposed (*i.e.*, open tube) filtration rate under the same input or recirculation rate. In other embodiments, the loss of leukocytes to the filtrate can be reduced by maintaining an input or recirculation rate that is greater than the filtration rate. In exemplary embodiments, the input or recirculation rate can be at least about five time, at least about 10 times, at least about 20 times, at least about 50 times, or at least about 100 times, the filtration rate.

A sample, or fluid mixture, comprising various blood constituents for cell fractionation by TFF can be obtained from a variety of sources and can include fluid mixtures of blood products at any of the various stages of processing. For example, blood sources can be either human or non-human. In addition, fluid mixtures can be, for example, whole blood,

various dilutions of whole blood, or whole blood or blood dilution that has been subjected to processing by, *e.g.*, removal of plasma or other blood constituents. Thus, the fluid mixture can include, for example, a blood cell population that is already at least partially enriched for leukocytes.

Blood constituents, or populations of leukocytes, can be prepared by methods known to those skilled in the art. Such methods typically include collecting heparinized blood, apheresis or leukopheresis, preparation of buffy coats, rosetting, centrifugation, density gradient centrifugation (*e.g.*, FICOLL-HYPAQUE®), PERCOLL®, sucrose, and the like), differential lysis of non-leukocyte cells, filtration, and the like. A leukocyte population can also be prepared by collecting blood from a subject, defibrinating to remove the platelets and lysing the majority of red blood cells. The population of leukocytes can optionally be enriched for monocytes by, for example, centrifugation through a PERCOLL® gradient.

The fluid mixture comprising the blood constituents can optionally be diluted or concentrated, as desired. For example, in certain embodiments, the blood constituents are diluted 1:2, 1:5, 1:10, or any other suitable dilution. Blood constituents can be diluted in, for example, isotonic buffers (*e.g.*, PBS or HEPES-buffered saline), tissue culture media and the like. Typically, the sample of blood constituents subjected to TFF has a cell concentration of about 10^6 to about 10^8 cells per ml.

Blood cell populations can be obtained from a variety of types of subjects, according to the desired use of the enriched population of leukocytes. The subject can be a healthy subject. Alternatively, blood cells can be obtained from a subject in need of immunostimulation, such as, for example, a cancer patient or other patient for which immunostimulation may be beneficial. Likewise, blood cells can be obtained from a subject in need of immune suppression, such as, for example, a patient having an autoimmune disorder (*e.g.*, rheumatoid arthritis, diabetes, lupus, multiple sclerosis, and the like). A blood cell population also can be obtained from an HLA-matched healthy individual for administration to an HLA-matched patient in need of immunostimulation. A blood cell population can also be collected from an individual that has been administered a stem cell mobilization agent such as

for example GM-CSF, G-CSF, AMD3100 (or other agent that inhibits CXCR-4 function), or low- or high-dose cyclophosphamide (Deliliers *et al.*, *Leuk. Lymphoma* 43:1957, 2002) and the like. The individual can be a patient that has received enriched cell population, a relative, or a HLA-matched individual.

In certain embodiments, the enriched population of leukocytes can be collected in the retentate, while other blood constituents pass into the filtrate. For example, for enrichment of a population of leukocytes (*e.g.*, including monocytes and lymphocytes), other blood constituents such as plasma, platelets, and/or erythrocytes can be among the constituents selectively removed into the filtrate. In additional embodiments, lymphocytes, or small lymphocytes, can be selectively removed and passed into the filtrate.

A tangential flow filtration device as depicted in Figures 9A through 9C typically comprises a cross-flow chamber (3) and a filtrate chamber (4). A filter (5) is positioned between and with one surface in fluid communication with the cross-flow chamber (the retentate surface) and other surface in fluid communication with the filtrate chamber (the filtrate surface). The cross-flow chamber, filtrate chamber and filter comprise a remover unit (1). In one embodiment, the cross-flow chamber typically has a volume of about 55 ml, and the filtrate chamber has a volume of about 25 ml. The filter diameter is typically substantially the same as the diameter of the cross-flow chamber. In certain embodiments used to demonstrate the utility of the present invention, the filter is about 140 mm to about 143 mm in diameter.

The fluid mixture enters the cross-flow chamber (3) through a fluid inlet (6) that is typically situated adjacent to the retentate surface of the filter and such that the fluid mixture (*e.g.*, sample) enters the chamber substantially parallel to the retentate surface of the filter. Typically, fluid is removed from the cross-flow chamber (3) through a fluid outlet (7), which is usually located at a portion of a cross-flow chamber perpendicular to the retentate surface of the filter. In certain exemplary embodiments, the cross-flow chamber inlet (6) diameter is about 7 mm to about 8 mm, and cross-flow chamber outlet (7) diameter is about 8 mm to about 10 mm. The filtrate is removed through an outlet (8) in the filtrate chamber (4).

Typically, the fluid mixture is introduced into the cross-flow chamber at a sufficient input rate such that the cross-flow of the fluid mixture across the surface of the filter (retentate surface) is at a velocity high enough to gently disrupt and back-mix fluid and cells at the contact surface of the filter, *i.e.*, the boundary layer. As used herein, "boundary layer" refers to that layer of fluid adjacent to and on the retentate side of the filter, typically left by fluid passing through the filter. This disruption of the boundary layer facilitates efficient filtration by preventing the material at the contact surface of the filter from binding to the filter or becoming stagnant, which can hinder efficient filtration. The input rate of the fluid mixture is usually not sufficient, however, to cause lysis of a substantial number of leukocytes.

In certain embodiments, the blood constituents are passed across the retentate surface of the filter by pumping the fluid mixture into the cross-flow chamber (3). The pump used to drive the cross-flow of fluid across the filter is referred to as the "cross-flow pump" or "recirculating pump" (14). The cross-flow pump can include any pumping device in fluid communication with the cross-flow chamber (3) sufficient to introduce the flow of fluid into the chamber and across the filter at the specified input rate, without causing substantial damage to the cells (*e.g.*, cell lysis). A cross-flow pump suitable for use in the present invention can include, *e.g.*, a peristaltic pump, piston pump, diaphragm pump, or roller pump. A peristaltic pump can be used, for example, where it is desired to maintain the TFF device as part of a "closed" system.

The fluid mixture is typically pumped into the cross-flow chamber (3) at an input rate that exceeds the filtration rate. In an exemplary embodiment, the input rate is about 1680 ml/minute, and the filtration rate is about 15 ml/minute. In other exemplary embodiments, the input rate is about 1600 to about 1800 ml/minute, and the filtration rate is about 10 to about 20 ml/minute. Non-leukocytic material (*e.g.*, erythrocytes, immune complexes, proteins, and the like) pass through the filter (5) into a filtrate chamber (4).

As discussed *supra*, the filtration rate is typically less than the unopposed (*i.e.*, open tube) rate. The filtration rate can be controlled, for example, by reducing or restricting the

size of the filtrate chamber outlet, by use of a second pump means (*e.g.*, a "filtration pump") to restrict the flow, and the like.

In another exemplary embodiment, the introduction of a fluid mixture into the device creates a vortex motion within the fluid. This can be done, for example, by introducing the fluid mixture, for example substantially parallel to a circular filter in a cylindrical cross-flow chamber, at an input rate about 5 or about 10 to about 100 times the filtration rate. The flow through is removed by means of an outlet (7) located in the cylindrical chamber perpendicular to the filter and typically adjacent to the center of the filter surface. This arrangement causes the flow to spiral inward toward the center of the filter. The flow is typically not turbulent, or at such a high rate, so as to cause substantial lysis of the leukocytes. As discussed above, the flow can also "scrub" the filter surface to prevent binding or stagnation at the boundary layer. By calibrating the input rate such that it is large (*e.g.*, at least about 5 times) relative to the filtration rate, the resulting enriched population of leukocytes can be at least about 20, or at least about 40 percent, or more, leukocytes.

In another exemplary embodiment, the retentate is recirculated to increase efficiency of separation. For example, a fluid mixture comprising blood constituents can be introduced into the cross-flow chamber, and then retentate can be withdrawn through the fluid outlet (7) in the cross-flow chamber to another chamber, such as, *e.g.*, a chamber from which the fluid was initially provided ("a recovery unit"; (2)). The fluid mixture in the recovery unit can then be re-introduced into the cross-flow unit. By connecting the recovery unit (2) and remover unit (1) in "loop format," continuous recirculation and filtration of the fluid mixture can be achieved. Alternatively, the retentate can be withdrawn through the fluid outlet (7) of the cross-flow chamber (3) and directly reintroduced into the cross-chamber inlet (*i.e.*, without passing through a recovery unit or another chamber). The fluid mixture can be passed through the cross-flow unit for any suitable period of time. In certain embodiments, the fluid mixture can be recirculated for about 5 to about 60 minutes, or more, to achieve the desired leukocyte cell purity or enrichment.

In yet another embodiment, the volume of the fluid mixture can be adjusted by adding a buffer, a wash solution or other solution (collectively referred to as a "replacement liquid"). The wash solution can, for example, be combined with a fluid mixture in a recovery unit (*e.g.*, through a solution inlet; (13)), in a remover unit, at a pump (14), in tubing extending to or from the remover unit, or at any other convenient location. The cells in the retentate can thus be enriched and washed in the same operation. Typically, the wash solution is isotonic with the cells. Suitable buffer and wash solutions can include a variety of buffers (*e.g.*, phosphate-buffered saline (PBS) or HEPES-buffered saline), tissue culture media, and the like.

In certain embodiments, cell populations are enriched for a population of leukocytes in a closed, aseptic system. As used herein, the terms "closed, aseptic system" or "closed system" refer to a system in which exposure to non-sterile, ambient, or circulating air or other non-sterile conditions is minimized or eliminated. Closed systems for enriching cell populations generally exclude centrifugation in open top tubes, open air transfer of cells, culture of cells in tissue culture plates or unsealed flasks, and the like. The entire filtration system, including, *e.g.*, any cell containers, incubators, tissue culture vessels, or other apparatus for cell processing (*infra*), can be maintained as a "closed" system. In a typical embodiment, the closed system allows aseptic enrichment of leukocytes and, optionally, transfer from an initial collection vessel to a sealable tissue culture vessel, without exposure to non-sterile air. Typically, a peristaltic pump (Figure 9A and 9C; (15)) means is used in a closed system.

In another tangential flow filtration procedure, a heterogeneous mixture of blood constituents is substantially enriched for leukocytes by the selective removal from the mixture of non-leukocyte blood constituents, including, *e.g.*, plasma, platelets, erythrocytes, and the like. As used herein, the term "substantially enriched" means that the cell population recovered in the retentate, following as many cycles of recirculation as desired, is comprised of at least about 20%, or at least about 40 %, or at least about 60%, of the desired cell type (*e.g.*, leukocytes). In other embodiments, a heterogeneous mixture of blood constituents is enriched for leukocytes to form an enriched population of leukocytes that is substantially free of non-leukocyte blood

constituents. As used herein, the term "substantially free" means that the enriched population of leukocytes comprises at least 50% leukocytes.

In an exemplary embodiment of this aspect, the TFF device comprises a cross-flow chamber (3) with a volume of about 55 ml and a filtrate chamber (4) with a volume of about 25 ml. Further the device comprised the following: a filter pore size of about 1 to about 10 microns, or about 2 to about 8 microns, or about 3 to about 5 microns; an input rate of about 1600 to about 1800 ml/min; a filtration rate of about 12 to about 17 ml/min, and a filter diameter of about 142 mm. The initial fluid mixture typically has a cell concentration of at least about 10^7 cells per ml (*e.g.*, leukocytes and other cells).

In another tangential filtration flow procedure, a heterogeneous mixture of blood constituents is substantially enriched for monocytes by the selective removal of non-monocyte blood constituents, including, for example, the removal of lymphocytes from the mixture. As used herein, the terms "selective removal," "selectively removed" and "selectively removing" refer to the preferential removal of one cell type and enriching for another cell type. In an exemplary embodiment of this aspect, the TFF device comprises a cross-flow chamber (3) with a volume of about 55 ml and a filtrate chamber (4) with a volume of about 25 ml. Further, the device comprised the following: a filter pore size of about 1 to about 10 microns, or about 2 to about 8 microns, or about 3 to about 5 microns; an input rate of about 1600 to about 1800 ml/min; a filtration rate of about 12 to about 17 ml/min; and a filter diameter of about 142 mm. The initial fluid mixture typically has a cell concentration of at least about 10^7 cells per ml (*e.g.*, monocytes and other cells). In this embodiment the device was operated in an inverted manner.

In yet another aspect of the invention, a heterogeneous mixture of blood constituents is substantially enriched for monocytic dendritic precursor cells. Following enrichment of a population of cells for leukocytes or monocytes, as described *supra*, monocytic dendritic cell precursors, such as those from peripheral blood, can be isolated from the enriched population through selective adherence to a substrate (*e.g.*, a monocytic dendritic cell precursor binding substrate). Such a substrate can be provided by, for example, a tissue culture dish or flask. Alternatively, a substrate having a high surface area to volume ratio, such as a particulate

or fibrous substrate, as disclosed in PCT/US02/23865, filed 25 July, 2002, the disclosure of which is incorporated by reference herein), can be used. The monocytic dendritic cell precursors can be monocytes that selectively adhere to the substrate to form complexes of monocytic dendritic cell precursors and substrate, while other leukocytes remain unbound ("non-adhering"). The bound leukocytes are then separated from the unbound leukocytes to form a population of cells enriched in monocytic dendritic cell precursors on the substrate. The monocytic dendritic cell precursors can be cultured and differentiated on the substrate, or eluted from the substrate and then cultured and differentiated separately, to obtain immature and/or mature, antigen-presenting dendritic cells. In accordance with this aspect, the monocytic dendritic cell precursors optionally can be isolated and differentiated in a closed, aseptic system. --

Please replace paragraph [0050] with the following amended paragraph:

-- In a particular embodiment, an antigenic peptide having an amino acid residue sequence Xaa Leu (or Met) Xaa Xaa Xaa Xaa Xaa Xaa Val (or Leu) (SEQ ID NO:3) (designated PSM-PX), where Xaa represents any amino acid residue, can be used as antigen. This peptide resembles the HLA-A0201 binding motif, *i.e.*, a binding motif of 9-10 amino acid residues with "anchor residues", leucine and valine found in HLA-A2 patients. (*See, e.g.*, Grey *et al.*, *Cancer Surveys* 22:37-49 (1995).) This peptide can be used as antigen for HLA-$A2^+$ patients (see, Central Data Analysis Committee "Allele Frequencies", Section 6.3, Tsuji, K. *et al.* (eds.), Tokyo University Press, pp. 1066-1077). Similarly, peptides resembling other HLA binding motifs can be used. --

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 - 2, at the end of the application.